



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:  
Didier Trono  
Patrick Salmon

Serial No.: 10/010,081

Filed: November 9, 2001

For: METHODS AND COMPOSITIONS  
RELATING TO IMPROVED  
LENTIVIRAL VECTORS AND THEIR  
APPLICATIONS

Group Art Unit: 1636

Examiner: Kaushal, Sumesh

Atty. Dkt. No.: CLFR:010US/DLP

**DECLARATION UNDER 35 U.S.C. §1.131**

We, Patrick Salmon and Didier Trono, declare as follows:

1. We are co-inventors of the subject matter disclosed and claimed in the referenced application.
2. We understand that the PTO Examiner examining our application has identified the scientific article of Ramezani *et al.*, *Mol. Therapy*, vol. 2, No. 5, November 2000, to be relevant to the patentability of various of the currently pending claims.
3. We are submitting this declaration to demonstrate that our invention, to the extent disclosed by the Ramezani *et al.* reference, was made by us in Switzerland at least as early as February 2000, the date that we submitted our research article entitled "High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors," *Blood*, Vol. 96, No. 10, pp 3392-3398, November 2000 (copy enclosed as Exhibit 1). As can be seen at the bottom of page 13 of the enclosed print out, the original manuscript was submitted on February 10, 2000. (Note that a copy of the



submitted manuscript is also enclosed as Exhibit 2.)

4. As can be seen from this publication and manuscript, they fully describe virtually all of the key aspects of the present invention, including the use of the SIN lentivector design employing various exemplary promoters determined to function well in human hematopoietic progenitors (the EF1 $\alpha$  and PGK promoters) in contrast to the prior art CMV promoter SIN constructs which were determined to function only poorly in these cells. The publication and manuscript also disclose constructs that employ a posttranslational regulatory element ("WPRES") in the section entitled "Influence of SIN design and WPRES on transgene expression." Thus, it is evident from these documents that we made our invention prior to the publication of Ramezani *et al.*


5. We further declare that we are the sole inventors of the subject matter disclosed and claimed in the present application, and that the other named co-authors of the Salmon *et al.* publication did not contribute to the conception of the presently claimed subject matter. Vincent Kindler was involved only in helping us purify the hematopoietic progenitors. Similarly, Odile Ducrey was involved in proceeding to some of the vector constructions and titrations, Bernard Chapuis and Rudolph Zubler were involved in providing expertise with hematopoietic stem cell isolation and purification. None of these individuals participated in the conception of the present invention.

6. We declare that all statements made herein of our own knowledge are true, and that all statements of our own belief are believed to be true, and further that these statements were made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under § 1001 of title 18 of the United States Code, and that such willful




false statements may jeopardize the validity of this patent, and any reexamination certificate issuing thereon.

Sept 10, 2004  
Date

  
Patrick Salmon

Sept. 8, 2004  
Date

  
Didier Trono



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Blood, 15 November 2000, Vol. 96, No. 10, pp. 3392-3398

GENE THERAPY

## High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors

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### ▶ Abstract

Recent experiments point to the great value of lentiviral vectors for the transduction of human hematopoietic stem cells (hHSCs). Vectors used so far, however, have been poorly satisfying in terms of either biosafety or efficiency of transgene expression. Herein is described the results obtained with human immunodeficiency virus-based vectors optimized in both of these aspects. It is thus shown that vectors containing the EF1 $\alpha$  and, to a lesser extent, the phosphoglycerate kinase (PGK) promoter, govern high-level gene expression in human hematopoietic progenitors as well as derived hematopoietic lineages of therapeutic relevance, such as erythrocytes, granulocytes, monocytes, dendritic cells, and megakaryocytes. EF1 $\alpha$  promoter-containing lentiviral vectors can also induce strong transgene expression in primary T lymphocytes isolated from peripheral blood. A self-inactivating design did not affect the performance of EF1 $\alpha$  promoter-based vectors but significantly reduced expression from the PGK promoter. This negative effect could nevertheless be largely rescued by inserting the post-transcriptional regulatory element of woodchuck hepatitis virus upstream of the vector 3' long terminal repeat. These results have important practical implications for the genetic treatment of lymphohematologic disorders as well as for the study of hematopoiesis via the lentivector-mediated modification of hHSCs. (Blood. 2000;96:3392-3398)

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## ► Introduction

Gene therapy via the transduction of human hematopoietic stem cells (hHSCs) represents a very promising approach for the treatment of a number of inherited and acquired lymphohematologic disorders. The stable genetic manipulation of long-term repopulating hHSCs with existing gene delivery systems, however, was until recently impossible to achieve at an efficiency compatible with therapeutic realities.

Oncoretroviral vectors derived from Moloney murine leukemia virus (MLV), for instance, although highly appealing because they integrate their cargo into the chromosomes of target cells, cannot transduce hHSCs that have not been first treated with inducers of proliferation.<sup>1,2</sup> Indeed, the nuclear transport of the MLV preintegration complex requires the breakdown of the nuclear envelope that occurs at mitosis.<sup>3,4</sup> Unfortunately hHSCs, whether harvested from the bone marrow, the umbilical cord, or mobilized in the peripheral circulation, are mostly quiescent and lose their pluripotentiality after stimulation and proliferation.<sup>5-7</sup> Recent reports, however, have shown that a significant fraction of pluripotent cells as well as cells capable of long-term engraftment in nonobese diabetic/severe combined immunodeficient (NOD/SCID) cells, also called SCID-repopulating cells (SRCs), can be maintained, transduced, and even expanded using specific stimulation conditions.<sup>7-10</sup>

Lentiviruses are a subgroup of retroviruses that can infect nondividing cells owing to the karyophilic properties of their preintegration complex, which allow for its active import through the nucleopore. Correspondingly, lentiviral vectors derived from human immunodeficiency virus type 1 (HIV-1) can mediate the efficient delivery, integration, and long-term expression of transgenes into nonmitotic cells both in vitro and in vivo.<sup>11-13</sup> In particular, HIV-based vectors can efficiently transduce human CD34<sup>+</sup> hematopoietic cells in the absence of cytokine stimulation,<sup>14-18</sup> and these cells are capable of long-term engraftment in NOD/SCID mice.<sup>17</sup> Furthermore, bone marrow from these primary recipients can repopulate secondary mice with transduced cells, confirming the lentivector-mediated genetic modification of very primitive hematopoietic precursors, most probably bona fide stem cells.<sup>36</sup> Because none of the other currently available gene delivery systems have such an ability, lentiviral vectors provide a previously unexplored basis for the study of hematopoiesis and for the gene therapy of inherited and acquired lymphohematopoietic disorders via the genetic modification of HSCs.

The demonstration of this important point, however, was provided with an early generation of lentiviral vectors unsuitable for therapeutic applications, either because they failed to meet biosafety requirements<sup>14-16</sup> or because they induced levels of transgene expression that were dismissingly low.<sup>17,18,20</sup> Our recent efforts have resulted in the development of lentiviral vectors that are improved in both of these aspects. Accordingly, the present article describes gene transfer vehicles that appear particularly well suited for the transduction of human hematopoietic precursor cells (HPCs) and for the expression of transgenes in differentiated blood lineages. Our

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results should facilitate the further use of lentiviral vectors for the genetic manipulation of lymphohematopoietic cells, be it for research or therapeutic purposes.

## ► Materials and methods

### Vectors

Production of MLV- and HIV-derived vectors pseudotyped with the vesicular stomatitis virus (VSV) G envelope protein was achieved by transient cotransfection of 3 plasmids into 293T epithelial cell line as previously described.<sup>11</sup> MLV vector particles were produced using the cytomegalovirus (CMV)-GagPol plasmid as packaging construct<sup>21</sup> and a vector derived from pSLX, which expressed green fluorescent protein (GFP) from the CMV promoter. The HIV-derived packaging construct used was pCMVR8.91, which encodes the HIV-1 Gag and Pol precursors as well as the regulatory proteins Tat and Rev.<sup>22</sup> VSV G was expressed from pMD.G. The HIV vector plasmids were derivatives of the original pHR' backbone,<sup>11</sup> with the following modifications. Self-inactivating (SIN) vectors were produced from the previously described SIN-18 vector, which contains a deletion in the U3 region of the 3' long terminal repeat (LTR) from nucleotide -418 to nucleotide -18, removing all the transcriptionally active sequences.<sup>23</sup> Insertion of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) between the reporter gene and the 3' LTR was done as previously described.<sup>24</sup> The phosphoglycerate kinase (PGK) promoter corresponded to nucleotides 424 to 930 of the murine PGK gene (GenBank accession number [M18735](#)). The EF1 $\alpha$  promoter was derived from the pEF-BOS plasmid<sup>25</sup> and comprised nucleotides 373 to 1561 of the human elongation factor EF1 $\alpha$  gene (GenBank accession number [J04617](#)). After transient transfection of the 3 plasmids by calcium phosphate in 293T cells, the supernatant was harvested, concentrated by 2 rounds of ultracentrifugation, and resuspended in serum-free medium (Cellgro SCGM, Cellgenix, Freiburg, Germany). Viral stocks were stored at -70°C and titers determined by transduction and flow cytometry analysis of GFP expression in HeLa cells as previously described.<sup>22</sup> Titers were composed of between  $5 \times 10^7$  and  $10^8$  HeLa-transducing units (TU) per milliliter.

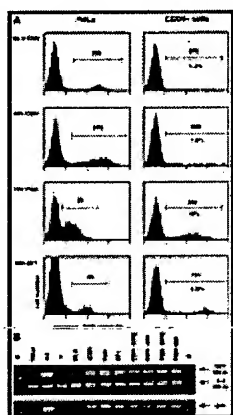
### Purification and transduction of CD34<sup>+</sup> cells

Cord blood samples were obtained according to institutional guidelines, and CD34<sup>+</sup> cells were purified as described.<sup>26</sup> In brief, cord blood mononuclear cells recovered after Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation were incubated on ice with anti-CD34 M450 Dynabeads (Dyna, Oslo, Norway) as described by the manufacturer. After several washes to eliminate unbound cells, CD34<sup>+</sup> cells were recovered from the beads by incubation for 15 minutes at 37°C with the "Detach-a-bead" included in the kit. Cells were immediately washed and analyzed by flow cytometry. The percentage of purified CD34<sup>+</sup> cells was  $89\% \pm 7.0\%$ . For transduction,  $10^5$  cells were seeded in 96-well plates in 100  $\mu$ L of Cellgro medium supplemented with antibiotics (Gibco BRL, Life Technologies Ltd, Paisley, Scotland) with  $10^{-4}$ -mol/L

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**Figure 1. Transgene transfer and expression after transduction of human CD34<sup>+</sup> cells with MLV and HIV vectors.** Human CD34<sup>+</sup> cells from umbilical cord blood (10<sup>5</sup> cells) were transduced with 10<sup>6</sup> HeLa-TU of MLV- or the indicated HIV-derived GFP encoding vectors, previously treated with DNase I, as described in "Materials and methods." After 4 days, cells were analyzed by flow cytometry for GFP expression (A) and lysed for PCR analysis of the presence of GFP transgene (B). Results from 1 experiment representative of 2 independent evaluations are shown. (A) Expression of the GFP transgene after transduction of human CD34<sup>+</sup> cells or HeLa cells. Results are represented as histograms of GFP fluorescence intensity (x-axis, 4-log scale) versus cell number (y-axis, linear). GFP<sup>+</sup> cells (within marker) were analyzed for percentage (lower number) and median of fluorescence intensity (upper number). Percentages were omitted in HeLa cells because the histograms were obtained from titration experiments of the corresponding vectors. (B) Presence of the GFP transgene after transduction and expansion of human CD34<sup>+</sup> cells. Cellular extracts equivalent to 5000 cells (upper panel) were amplified as described in "Materials and methods" with GFP-specific primers, together with IL-2-specific primers as internal controls. Sizes of the corresponding PCR products are indicated. Lanes are as follows: M, molecular weight marker; HeLa (negative control for GFP, positive control for IL-2); 4.5, a clone of HeLa containing 1 copy of HIV-CMV-GFP vector; 0, untransduced CD34<sup>+</sup> cells; MLV-CMV-PGK-EF1, CD34<sup>+</sup> cells transduced with the corresponding vectors (see A); CMV-EPO, CD34<sup>+</sup> cells transduced with HIV-CMV vector (lane CMV) and analyzed after expansion and differentiation into erythroid cells (see text); CMV-GM, same as CMV-EPO but analyzed after expansion and differentiation into monocytic cells (see text); PGK-EPO and



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PGK-GM, same as CMV-EPO and CMV-GM but from CD34<sup>+</sup> cells transduced with HIV-CMV vector (lane PGK). To ensure proportionality, cellular extracts equivalent to 1700 cells (lower panel) were amplified separately.

### PCR analysis after transduction of CD34<sup>+</sup> cells

Cells ( $5 \times 10^5$ ) were lysed in 18  $\mu$ L of 50-mmol/L Tris HCl, pH 8.0; 20-mmol/L NaCl; 1-mmol/L ethylenediaminetetraacetic acid; and 1% sodium dodecyl sulfate. Proteinase K (2  $\mu$ L at 10 mg/mL) was added, and samples were incubated for 30 minutes at 55°C. After digestion, 200  $\mu$ L of water was added, and samples were boiled for 10 minutes to inactivate proteinase K. PCR amplification was performed using HotStarTaq (Qiagen GmbH, Hilden, Germany) in a final volume of 50  $\mu$ L and 2  $\mu$ L or 0.7  $\mu$ L of extract with the following cycles: 95°C, 15 minutes; 40 cycles 94°C, 30 minutes; 60°C, 1 minute; 72°C, 1 minute; plus a 10-minute extension phase at 72°C. Sequences of the PCR primers were as follows: 5' human interleukin-2 (hIL-2) GCAACTCCTGTCTTGCATTG; 3' hIL-2 AATGTGAGCATCCTGGTGAG; 5' GFP GTGAGCAAGGGCGAGGAGC; and 3' GFP CTTGATGCCGTTCTTCTGCTTGT. Conditions were adjusted to observe a decrease in the final PCR product yield when diluting samples by a factor of 3 (Figure 1A).

### Purification and transduction of primary human T cells

Peripheral blood mononuclear cells were purified from buffy coats of healthy donors over a Ficoll-Paque gradient. Macrophages were removed by plastic adherence for 2 hours at 37°C, and the remaining cells were incubated with a cocktail of monoclonal antibodies against HLA-DR, CD25, CD69, CD19, CD16, CD11b, and CD14 (Pharmingen, San Diego, CA). After a 30-minute incubation on ice, cells were washed twice and incubated with magnetic beads (Dynabeads) conjugated with goat antimouse immunoglobulin (Ig) G at a 1:4 target:bead ratio. Bead-bound cells were removed 30 minutes later using a magnet. Remaining cells were further purified through 2 more rounds at an increased target:bead ratio (1:10). This purification protocol typically resulted in a 99.5% pure population of resting T cells as determined by flow cytometry analysis with antibodies against the activation markers HLA-DR, CD25, and CD69. Cells maintained in RPMI 1640 supplemented with 10% fetal calf serum, 5-mmol/L penicillin and streptomycin (GIBCO BRL), and 2-mmol/L glutamine (GIBCO BRL) were activated with phytohemagglutinin (PHA; Sigma, St Louis, MO) at 3  $\mu$ g/mL for 48 hours and subsequently cultured in RPMI 1640 containing 10% fetal calf serum and recombinant IL-2 (Sigma) at 10 U/mL. A total of  $5 \times 10^5$  cells were transduced with various lentiviral vectors at a multiplicity of infection (MOI) of 5 HeLa-TU per cell in 24-well plates in the presence of PHA, in a final volume of 500  $\mu$ L. Flow cytometry analysis of GFP expression was performed 5 days after transduction.

### Cytokines

All cytokines were recombinant human material. Granulocyte-macrophage colony-stimulating



factor (GM-CSF; Leucomax) from Essex Chimie & Sandoz (Basel, Switzerland) was used at 20 ng/mL, G-CSF (Neupogen) from Roche (Basel, Switzerland) at 10 ng/mL, and erythropoietin (Eprex) from Cilag (Schaffhausen, Switzerland) at 2 U/mL. Other cytokines were purchased from Peprotech EC (London, England) and used at the following concentrations: FLT3-L 25 ng/mL, TPO 10 U/mL, SCF 20 ng/mL, IL-3 10 ng/mL, tumor necrosis factor (TNF) 40 ng/mL, and IL-4 20 ng/mL.

### **Antibodies and immunoreactants**

Phycoerythrin-conjugated monoclonal antibodies (mAbs) were the following: anti-CD34 (mIgG1 clone 8G12) from Becton Dickinson (Mountain View, CA), anti-glycophorin-A (mIgG1, clone JC 159), anti-CD42b (mIgG2a, clone AN51), and isotypic controls from Dako (Glostrup, Denmark). Biotin-labeled mAbs included anti-CD14 (mIgG2a, clone UCHM1) from Ancell Corp (Bayport, MN), anti-CD15 (mIgM clone DU-HL60-3) from Sigma, and isotypic controls from Ancell. Allophycocyanin-labeled streptavidin was from Pharmingen. Anti-CD34 mIgG-coated M450 Dynabeads were from Dynal.

### **In vitro differentiation**

Megakaryocytic differentiation was evaluated after 10 days of expansion culture with FLT3-L, TPO, and SCF. For the other lineages, cells were washed after 9 to 11 days of expansion culture with FLT3-L, TPO, and SCF and incubated with erythropoietin (EPO), IL-3, and SCF for erythroid differentiation, GM-CSF and SCF for monocytic differentiation, and G-CSF and SCF for granulocytic differentiation. Differentiation into dendritic cells (DCs) was performed as previously described.<sup>26</sup> Briefly, transduced CD34<sup>+</sup> cells were expanded for 14 to 28 days with FLT3-L, TPO, and SCF. Cells were then induced into mature DCs by exposure to GM-CSF and IL-4 for 3 days, followed by GM-CSF, IL-4, and TNF for 3 more days. The amplification ratio was between 100 and 1000, depending on the experiment and the cell lineage.

### **Flow cytometry analysis**

Cells were analyzed as described<sup>26</sup> on a FACSCalibur (Becton Dickinson) with slight modifications. FL-1 was used for GFP, FL-2 for phycoerythrin-labeled mAbs, FL-3 for identification of living cells with the nonpermeant DNA dye 7-amino-actinomycin D (Sigma),<sup>27</sup> and FL-4 for biotinylated mAbs indirectly labeled with streptavidin-allophycocyanin. Cell suspensions were adjusted to 0.5% paraformaldehyde prior to analysis. Data were analyzed using WINMDI software written by J. Trotter at Scripps Institute (La Jolla, CA) and CellQuest software (Becton Dickinson).

## **► Results**

**Transduction of human hematopoietic progenitors with MLV vectors and HIV-based lentivectors: the EF1 $\alpha$  promoter induces**

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## high transgene expression in lentivector-transduced CD34<sup>+</sup> cells

### ▲ Materials and methods

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Human cord blood CD34<sup>+</sup> cells were purified by a single round of positive selection with magnetic beads, resulting in preparations approximately 90% pure. Cells were maintained overnight in serum-free medium supplemented with TPO and were exposed for 24 hours to MLV- or HIV-based GFP-expressing vectors pseudotyped with VSV G prepared by transient transfection of 293T cells as previously described.<sup>11,22</sup> The lentiviral packaging system used in these experiments was of a so-called second generation<sup>22</sup> and included the HIV-1 *gag*, *pol*, *tat*, and *rev* genes. The MOI was 10 HeLa-TU per CD34<sup>+</sup> cell. After transduction, cells were incubated for another 3 days in medium containing fetal calf serum, FLT3-L, TPO, and SCF and analyzed by flow cytometry for GFP (Figure 1) and CD34 expression (not illustrated) and lysed for PCR analysis. The relative potencies of 3 internal transcription units were compared within the context of lentivectors: the CMV, the PGK, and the EF1 $\alpha$  promoters.

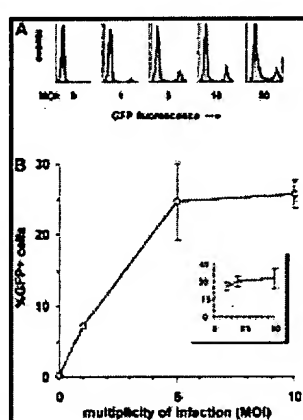
As shown in Figure 1A, a sharp subpopulation of GFP<sup>+</sup> hematopoietic progenitors could only be seen when cells were transduced with HIV vectors containing the PGK or the EF1 $\alpha$  promoters. Cells transduced with MLV vector or HIV vector containing the CMV promoter displayed only a small percentage of GFP<sup>+</sup> cells together with a high heterogeneity in GFP expression. A side-by-side comparison of GFP expression after transduction of HeLa and CD34<sup>+</sup> cells revealed that the promoters examined behaved quite differently in these 2 cell types. The PGK promoter was weak in HeLa cells (overlap between GFP<sup>+</sup> and GFP<sup>-</sup> cells) and strong in HPCs. The EF1 $\alpha$  promoter was intermediate in HeLa cells and very potent in HPCs, with a mean value typically 100 times higher in transduced than in control HPCs. The CMV promoter, whether in an MLV or an HIV vector, was equally strong in HeLa cells, contrasting with its low activity in transduced HPCs. A PCR-based quantification performed at the time of the flow cytometry analysis (Figure 1B) indicated that the low expression from MLV-CMV vector in HPCs was due at least in part to poor gene transfer in these cells, a consequence of the limited stimulation of CD34<sup>+</sup> cells during transduction. In contrast, the low expression in HPCs exposed to the HIV-CMV vector (Figure 1A) could not be explained by a low frequency of transduction, because equivalent amounts of transgene DNA were detected in cells transduced with all 3 HIV vectors (Figure 1B). This indicates that the CMV promoter does not govern efficient expression in hematopoietic progenitors, a finding in accordance with previous reports.<sup>17,18,20</sup> We also ascertained that GFP expression originated from integrated proviruses. For this, we performed a PCR analysis following 3 weeks of expansion of the transduced CD34<sup>+</sup> cells. During this period, the cell numbers increased by a factor of 900 to 1800, depending on conditions and experiments. In the particular experiment illustrated here (Figure 1B), the percentage of cells expressing GFP from the PGK promoter decreases by a factor of approximately 2 after expansion and differentiation into erythroid or monocytoid cells, which paralleled a slight decrease in GFP DNA copy number. It confirms that the bulk of transgene DNA remains in the cells after massive expansion, excluding its presence as unintegrated forms. It also correlates the previous demonstration that long-term transgene expression cannot be detected after transduction with integrase-deficient



HIV vectors.<sup>12</sup>

### Transduction of human CD34<sup>+</sup> cells as a function of increasing vector concentration

The high levels of GFP expression induced by the EF1 $\alpha$ -containing HIV-derived vector allowed for a reliable determination of gene transfer efficiency because even low numbers of transduced cells were easily detected. This vector was thus used to evaluate the influence of the MOI on the transduction efficiency of CD34<sup>+</sup> cell (Figure 2). Although the percentage of GFP<sup>+</sup> cells initially increased as a direct function of the MOI, the curve flattened starting at an MOI of 5, reaching a maximum of 25% GFP<sup>+</sup> cells ( $\pm 5\%$  according to experiments) at MOIs 20 and above. In the absence of optimal cytokine-induced proliferation, only a fraction of human CD34<sup>+</sup> cells is thus permissive to lentivector-mediated transduction, as previously suggested.<sup>28</sup>



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**Figure 2. Effect of MOI on transduction efficiency of lentivectors in human CD34<sup>+</sup> cells.** A total of  $10^5$  CD34<sup>+</sup> cells were transduced with various doses (1, 2, 5, 10, 20, and  $50 \times 10^5$  TU) of EF1 $\alpha$ -GFP lentiviral vector, allowing MOIs ranging from 1 to 50. GFP expression was analyzed by flow cytometry after 4 days. (A) Data from one representative experiment are shown as frequency histograms of GFP fluorescence intensity versus cell number (events). Gates for GFP<sup>+</sup> cells were set up according to untransfected cells (MOI 0). (B) Mean percentages of GFP<sup>+</sup> cells obtained with MOIs of 0 to 10 and 10 to 50 are represented in the main panel and in the insert, respectively. These data are from 3 independent experiments for the 0-to-10 MOI range and from 2 independent experiments for the 10-to-50 MOI range. Error bars represent SD.

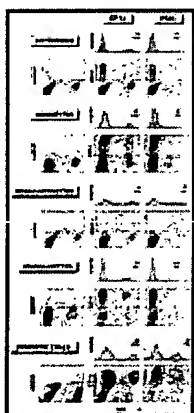
### Transgene expression in hematopoietic lineages after transduction of CD34<sup>+</sup> cells with HIV vectors containing PGK and EF1 $\alpha$ promoters

A critical issue for the genetic treatment of a variety of lymphohematologic disorders will be the levels of expression of the putatively therapeutic transgene in the appropriate subset of differentiated cells. In that respect, recent reports using HIV-based vectors with an internal expression cassette driven by the CMV promoter have documented the incapacity of this transcriptional element to induce strong transgene expression in precursors as well as mature blood cells.<sup>17,18</sup> With the hope of identifying more suitable alternatives, hematopoietic progenitors transduced with HIV-based vectors containing the PGK or the EF1 $\alpha$  promoters were differentiated in vitro to compare the relative potency of these vectors in various hematopoietic lineages. For this, transduced CD34<sup>+</sup> cells were first expanded for 7 to 14 days in FLT3-L/TPO/SCF and then incubated in various differentiating media for an additional 5- to 10-day period, allowing the generation of erythroid cells, granulocytes, and monocytes (see "Materials



and methods"). Megakaryocytes were induced in FLT3-L/TPO/SCF exclusively. For DCs, transduced CD34<sup>+</sup> cells were expanded for 14 to 28 days with FLT3-L/TPO/SCF. Cells were then induced into mature DCs by exposure to GM-CSF/IL-4 for 3 days, followed by exposure to GM-CSF/IL-4/TNF for 3 more days.

The differentiated cells were then analyzed by flow cytometry to determine the percentage of lineage-specific marker<sup>+</sup>/GFP<sup>+</sup> cells as well as the relative levels of GFP expression in the differentiated populations (Figure 3). Transgene expression was high in all lineages examined after transduction of precursors with the EF1 $\alpha$ -containing lentiviral vector, with a signal-to-noise ratio (mean of fluorescence intensity of GFP<sup>+</sup> cells divided by mean of fluorescence intensity of GFP<sup>-</sup> cells) between 150 and 200, depending on the cell type. The PGK promoter induced lower and more variable levels of GFP expression in differentiated cells. Expression of GFP under the control of the PGK promoter was highest in DCs, where it was only 3.5-fold less potent than that driven from the EF1 $\alpha$  promoter, and worst in erythroid cells (glycophorin<sup>+</sup>), where it was 11-fold weaker than its EF1 $\alpha$  counterpart. However, even in this case, the signal-to-noise ratio was still high enough to permit a clear discrimination between GFP<sup>+</sup> and GFP<sup>-</sup> cells, allowing for the reliable analysis of transgene expression.



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**Figure 3. Transgene expression in differentiated hematopoietic lineages after transduction of human CD34<sup>+</sup> cells with HIV-derived vectors.** Human CD34<sup>+</sup> cells from umbilical cord blood ( $10^5$  cells) were transduced with  $10^6$  TU of HIV vectors (corresponding to an MOI of 10) containing either the EF1 $\alpha$  or the PGK promoters and differentiated into various hematopoietic lineages as described in "Materials and methods." After differentiation, HIV-EF1 $\alpha$ - (first and second column) or HIV-PGK-transduced cells (third column) were analyzed by flow cytometry for both GFP (x-axis) and lineage-specific marker expression (glycophorin, CD14, CD42b, CD15, and CD1a, respectively, for erythroids, monocytes, megakaryocytes, granulocytes, and DCs). Isotype control antibodies were used in the first column. For each promoter, cell populations expressing high levels of lineage-specific markers were gated (upper rectangle in 2-dimensional plots), and monoparametric frequency histograms of GFP expression for these cells were generated (located above the 2-dimensional plots). The percentage of GFP<sup>+</sup> cells (upper number) and median of fluorescence intensity of GFP (lower number) was determined. For the experiment generating erythroids, monocytes, megakaryocytes, and granulocytes, the fraction of GFP<sup>+</sup> CD34<sup>+</sup> cells before differentiation was 22% for EF1 and 16% for PGK. For the experiment generating DCs, the fraction of GFP<sup>+</sup> precursors before differentiation was 32% for EF1 and 29% for PGK. These data are representative of 4 independent experiments.

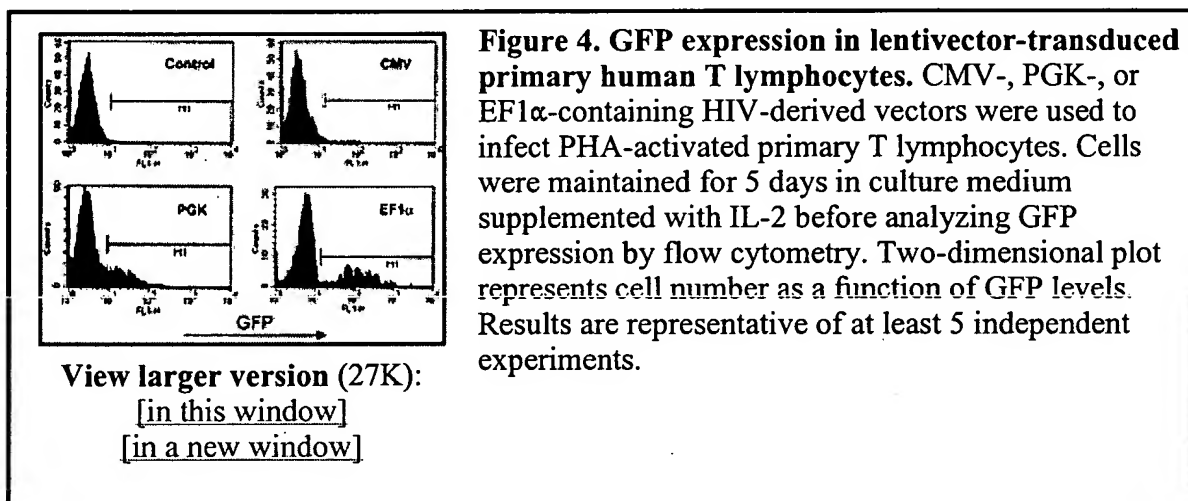
Taken together, these data indicate that the transduction of human CD34<sup>+</sup> cells with HIV-based vectors containing internal promoters derived from the EF1 $\alpha$  and, to a lesser extent, the PGK



gene results in high levels of transgene expression in several hematopoietic lineages.

### EF1 $\alpha$ promoter-based lentiviral vectors induce high levels of transgene expression in primary T lymphocytes

The system used in these experiments did not allow for the easy differentiation of CD34<sup>+</sup> precursors into cells of the lymphocytic lineage. Therefore, to compare the relative value of CMV-, PGK-, and EF1 $\alpha$ -containing HIV-derived vectors in these cells, mature T lymphocytes purified from the peripheral blood were directly used as transduction targets (Figure 4). The results revealed the same hierarchy as observed in HPCs and in the various other blood cell lineages, with the EF1 $\alpha$  promoter inducing levels of GFP expression significantly higher than those yielded by the PGK promoter, whereas cells transduced with CMV-based vectors exhibited levels of GFP expression that were incompatible with proper detection by flow cytometry. Of note, preliminary experiments in human primary B cells show that the CMV promoter is very active in these cells (not illustrated). This indicates that the poor activity of the CMV promoter is not universal in human hematopoietic cells.

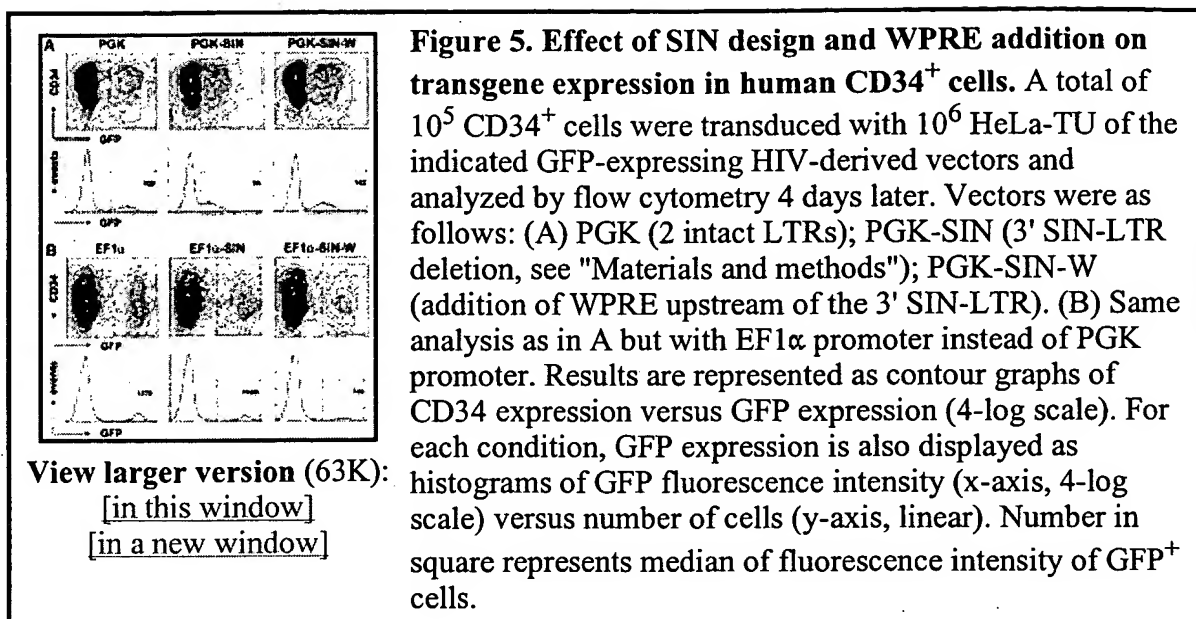


### Influence of SIN design and WPRE on transgene expression

The use of SIN 3' LTR U3-deleted HIV vectors increases the biosafety of this system and avoids a possible interference between the viral LTR and the vector's internal promoter.<sup>23</sup> EF1 $\alpha$ - and PGK-containing HIV-derived SIN vectors were therefore tested for their ability to induce high levels of transgene expression in hematopoietic precursors (Figure 5). Most interestingly, the SIN design was accompanied by a dramatic decrease (6-fold) in GFP expression within the context of the PGK vector, whereas it instead had a slightly yet reproducibly positive effect when introduced in the EF1 $\alpha$  vector. Inserting the sequence for the WPRE<sup>29</sup> upstream of the 3' LTR has been shown to promote transgene expression from HIV-derived vectors in some targets.<sup>24</sup> It indeed stimulated GFP production from the PGK promoter by a factor of 2.15 in hematopoietic precursors. In contrast, it exerted a negative effect on expression from the highly active EF1 $\alpha$  promoter, with a decrease by a factor of 1.85. Taken together, these indicate that the strong EF1 $\alpha$  promoter is less sensitive than the PGK promoter to a SIN configuration, making it a better



candidate for gene expression in hematopoietic cells.



## ► Discussion

This work describes the transduction of human CD34<sup>+</sup> cells with improved HIV-derived vectors aimed at increasing gene expression as well as biosafety. Our results indicate that these vectors can efficiently transduce CD34<sup>+</sup> cells using conditions under which MLV-based vectors are inefficient. Furthermore, our study demonstrates that an HIV-derived vector containing an internal EF1 $\alpha$  promoter induces high levels of transgene expression in both hematopoietic precursors and in vitro differentiated blood lineages, and in primary T cells. Using this vector, we also found that human CD34<sup>+</sup> cells can be efficiently transduced at a relatively low MOI but that the efficacy of gene transfer saturates at about 20% to 30% of transduced cells. Moreover, the HIV-derived vector containing an internal EF1 $\alpha$  promoter fully tolerates a SIN design.

Lentiviral vectors represent a very attractive tool for gene therapy of hematologic disorders because of their ability to transduce pluripotent hHSCs.<sup>14-18</sup> Two essential issues will need, however, to be addressed prior to envisaging the use of HIV-based vectors for therapeutic purposes. The first issue is biosafety, to which the use of multiply attenuated packaging constructs, stable producer cell lines, and SIN vectors will significantly reduce the risk that replication-competent recombinants emerge from the vector-manufacturing system. The second issue is efficiency, ie, the levels of transgene expression in the therapeutically relevant mature blood cell. Indeed, for instance, with hematologic disorders resulting from an enzymatic deficiency or with anemias secondary to defects in hemoglobin synthesis, function will not be restored without achieving significantly high levels of expression of the curative gene. In that

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respect, GFP represents a marker of choice to study promoter activity in target cells because it permits a direct quantification of the percentage of transduced cells as well as of the level of transgene expression in each individual cell.

CMV promoter-containing HIV-derived vectors can induce high levels of transgene expression in the central nervous system<sup>11-13</sup> and have allowed the initial demonstration that pluripotent hematopoietic precursors can be efficiently transduced by this gene delivery tool. CMV-based vectors, however, are largely useless for transferring therapeutic genes into most lymphohematopoietic cells because in these targets their transcriptional activity is prohibitively low.<sup>17,18,20</sup> We thus observed that CMV-driven GFP production was insufficient to determine reliably either the percentage of transduced cells or the level of transgene expression in both CD34<sup>+</sup> cells and several differentiated hematopoietic derivatives (Figures 1 and 4, and data not shown). In sharp contrast, vectors in which GFP was transcribed from the EF1 $\alpha$  or, to a lesser extent, the PGK promoter were highly active in both categories of cells.

The EF1 $\alpha$ -GFP lentivector was used to assess the dose responsiveness of CD34<sup>+</sup> cell transduction because GFP<sup>+</sup> cells could easily be discriminated from GFP<sup>-</sup> cells even when present in very low amounts. Surprisingly, under conditions of limited stimulation, we found that increasing the MOI above 10 did not increase significantly the fraction of transduced cells, which plateaued between 20% to 30%, depending on the experiments. A similar saturation of the fraction of transducible CD34<sup>+</sup> cells was previously noted and attributed to the fact that lentivectors can transfer genes into cells that are in the G<sub>1</sub> but not in the G<sub>0</sub> phase of the cycle.<sup>28</sup> Still, lentivector-transduced CD34<sup>+</sup> cells are pluripotent.<sup>17,19</sup> It remains to be determined whether the fraction of permissive hHSCs can be augmented, without triggering cell division, by the use of specific cocktails of cytokines, as recently described for the transduction of resting human T cells with HIV vectors.<sup>30</sup> Alternatively, transduction of human CD34<sup>+</sup> cells could also be improved under conditions promoting cell division, as recently reported.<sup>7,10</sup>

The MOI of 10 used here to achieve optimal transduction is significantly lower than the ones described in previous studies, where it ranged between 60 to 300 and 1000 to 3000.<sup>17,18</sup> This difference may be partly artifactual, because we define the titer of HIV vector stocks in HeLa cells, which are approximately 4 times less sensitive to transduction than 293T cells, another commonly used target for lentivector titration (data not shown). Nevertheless, it is also worth emphasizing that we expose the CD34<sup>+</sup> cells to the vector particles in a small volume (10<sup>5</sup> cells in 200  $\mu$ L) and during 24 hours—2 factors that could enhance the vector-target meeting probability.

Both HIV-PGK and HIV-EF1 $\alpha$  vectors were analyzed for transgene expression in differentiated hematopoietic cells after transduction of CD34<sup>+</sup> cells. GFP could be readily detected in all lineages analyzed, after transduction with both types of vectors. However, transgene expression in lineage marker-positive cells was consistently higher with the EF1 $\alpha$  promoter than with its PGK counterpart. Also, there was a lower degree of interlineage variation with the EF1 $\alpha$  promoter. It is remarkable that, with both of these promoters, the percentage of GFP<sup>+</sup> cells



remained fairly constant as the CD34<sup>+</sup> cells proliferated (global increase in cell number was between 100 and 1000; Figure 3) and differentiated into their various hematopoietic derivatives. This indicates that there was neither silencing of the promoters nor preferential loss of transduced cells during the expansion and maturation phases of the culture. Engraftment and repopulation assays in NOD/SCID will be performed with both HIV-PGK and HIV-EF1 $\alpha$  vectors to confirm the stability of expression from these promoters in vivo.

Lastly, we studied the effect of 2 modifications in the HIV vector backbone designed to improve its utility as a therapeutic tool. One is the deletion of a major part of U3 in the 3' LTR of the vector plasmid, leading to a SIN configuration.<sup>23</sup> This deletion reduces the potential for generation of replication-competent viruses and prevents potential interference between the LTR and internal promoter. In some circumstances, however, it can negatively affect transgene expression, apparently by decreasing the efficiency of polyadenylation.<sup>31-36</sup> We found that the SIN design exerted a dramatically negative impact on GFP expression in CD34<sup>+</sup> cells transduced with a PGK-based lentivector, while it did not decrease transcription efficiency from the EF1 $\alpha$  promoter. It is possible that the SIN deletion may be deleterious only in the context of low levels of transcription.

The SIN-induced decrease in PGK-driven transgene expression could be partly rescued by inserting the WPRE in the vector—immediately upstream of the deleted 3' LTR. This confirms our recent demonstration that WPRE can stimulate gene expression by a factor of 3 to 6 in epithelial cells.<sup>24</sup> However, the addition of WPRE did not stimulate GFP expression in CD34<sup>+</sup> cells transduced with an EF1 $\alpha$  promoter-based vector, having instead a slight yet reproducible negative effect. Although our lack of understanding of the mechanism of action of WPRE makes it difficult to explain this difference, it could be that the high amounts of transcripts generated by the EF1 $\alpha$  promoter clog the pathway targeted by this post-transcriptional regulatory element.

In conclusion, the present work indicates that EF1 $\alpha$  promoter-containing HIV-derived vectors are both highly efficient and very potent for expressing transgenes in human hematopoietic progenitor cells as well as in all of their blood cell derivatives, even in a SIN configuration. These vectors thus represent attractive tools for the genetic treatment of lymphohematologic disorders and for the study of hematopoiesis via the lentivector-mediated modification of human HSCs.

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## ► Footnotes

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P.S. and V.K. contributed equally to this work.

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## ► References

1. Kohn DB, Nolta JA, Weinthal J, et al. Toward gene therapy for Gaucher disease. *Hum Gene Ther.* 1991;2:101-105[[Medline](#)] [[Order article via Infotrieve](#)].
2. Mazurier F, Moreau-Gaudry F, Maguer-Satta V, et al. Rapid analysis and efficient selection of human transduced primitive hematopoietic cells using the humanized S65T green fluorescent protein. *Gene Ther.* 1998;5:556-562[[Medline](#)] [[Order article via Infotrieve](#)].
3. Roe T, Reynolds TC, Yu G, Brown PO. Integration of murine leukemia virus DNA depends on mitosis. *EMBO J.* 1993;12:2099-2108[[Abstract](#)].
4. Lewis PF, Emerman M. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol.* 1994;68:510-516[[Abstract](#)].
5. Bhatia M, Bonnet D, Kapp U, Wang JC, Murdoch B, Dick JE. Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. *J Exp Med.* 1997;186:619-624[[Abstract/Free Full Text](#)].
6. Dao MA, Hannum CH, Kohn DB, Nolta JA. FLT3 ligand preserves the ability of human CD34+ progenitors to sustain long-term hematopoiesis in immune-deficient mice after ex vivo retroviral-mediated transduction. *Blood.* 1997;89:446-456[[Abstract/Free Full Text](#)].
7. Dorrell C, Gan OI, Pereira DS, Hawley RG, Dick JE. Expansion of human cord blood CD34(+)CD38(-) cells in ex vivo culture during retroviral transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. *Blood.* 2000;95:102-110[[Abstract/Free Full Text](#)].
8. Dao MA, Hashino K, Kato I, Nolta JA. Adhesion to fibronectin maintains regenerative capacity during ex vivo culture and transduction of human hematopoietic stem and progenitor cells. *Blood.* 1998;92:4612-4621[[Abstract/Free Full Text](#)].

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9. Piacibello W, Sanavio F, Severino A, et al. Engraftment in nonobese diabetic severe combined immunodeficient mice of human CD34(+) cord blood cells after ex vivo expansion: evidence for the amplification and self-renewal of repopulating stem cells. *Blood*. 1999;93:3736-3749 [[Abstract/Free Full Text](#)].
10. Ueda T, Tsuji K, Yoshino H, et al. Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. *J Clin Invest*. 2000;105:1013-1021 [[Abstract/Free Full Text](#)].
11. Naldini L, Blomer U, Gallay P, et al. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science*. 1996;272:263-267 [[Abstract](#)].
12. Naldini L, Blomer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A*. 1996;93:11382-11388 [[Abstract/Free Full Text](#)].
13. Blomer U, Naldini L, Kafri T, Trono D, Verma IM, Gage FH. Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J Virol*. 1997;71:6641-6649 [[Abstract](#)].
14. Akkina RK, Walton RM, Chen ML, Li QX, Planelles V, Chen IS. High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *J Virol*. 1996;70:2581-2585 [[Abstract](#)].
15. Sutton RE, Wu HT, Rigg R, Bohnlein E, Brown PO. Human immunodeficiency virus type 1 vectors efficiently transduce human hematopoietic stem cells. *J Virol*. 1998;72:5781-5788 [[Abstract/Free Full Text](#)].
16. Uchida N, Sutton RE, Frier AM, et al. HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. *Proc Natl Acad Sci U S A*. 1998;95:11939-11944 [[Abstract/Free Full Text](#)].
17. Miyoshi H, Smith KA, Mosier DE, Verma IM, Torbett BE. Transduction of human CD34+ cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science*. 1999;283:682-686 [[Abstract/Free Full Text](#)].
18. Case SS, Price MA, Jordan CT, et al. Stable transduction of quiescent CD34(+)CD38(-) human hematopoietic cells by HIV-1-based lentiviral vectors. *Proc Natl Acad Sci U S A*. 1999;96:2988-2993 [[Abstract/Free Full Text](#)].
19. Woods N-B, Fahlman C, Mikkola H, et al. Lentiviral gene transfer into primary and secondary NOD/SCID repopulating cells. *Blood*. 2000;96:3725-3733 [[Abstract/Free Full Text](#)].
20. An DS, Wersto RP, Agricola BA, et al. Marking and gene expression by a lentivirus vector in transplanted human and nonhuman primate CD34(+) cells. *J Virol*. 2000;74:1286-1295 [[Abstract/Free Full Text](#)].
21. Scharfmann R, Axelrod JH, Verma IM. Long-term in vivo expression of retrovirus-mediated gene transfer in mouse fibroblast implants. *Proc Natl Acad Sci U S A*. 1991;88:4626-4630 [[Abstract](#)].



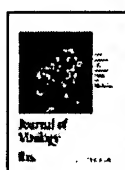
22. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol.* 1997;15:871-875[[Medline](#)] [[Order article via Infotrieve](#)].
23. Zufferey R, Dull T, Mandel RJ, et al. Self-inactivating lentivirus vector for safe and efficient In vivo gene delivery. *J Virol.* 1998;72:9873-9880[[Abstract/Free Full Text](#)].
24. Zufferey R, Donello JE, Trono D, Hope TJ. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol.* 1999;73:2886-2892[[Abstract/Free Full Text](#)].
25. Mizushima S, Nagata S. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* 1990;18:5322[[Medline](#)] [[Order article via Infotrieve](#)].
26. Arrighi JF, Hauser C, Chapuis B, Zubler RH, Kindler V. Long-term culture of human CD34 (+) progenitors with FLT3-ligand, thrombopoietin, and stem cell factor induces extensive amplification of a CD34(-)CD14(-) and a CD34(-)CD14(+) dendritic cell precursor. *Blood.* 1999;93:2244-2252[[Abstract/Free Full Text](#)].
27. Schmid I, Uittenbogaart CH, Keld B, Giorgi JV. A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. *J Immunol Methods.* 1994;170:145-157[[Medline](#)] [[Order article via Infotrieve](#)].
28. Sutton RE, Reitsma MJ, Uchida N, Brown PO. Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. *J Virol.* 1999;73:3649-3660[[Abstract/Free Full Text](#)].
29. Donello JE, Loeb JE, Hope TJ. Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element. *J Virol.* 1998;72:5085-5092[[Abstract/Free Full Text](#)].
30. Unutmaz D, KewalRamani VN, Marmon S, Littman DR. Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. *J Exp Med.* 1999;189:1735-1746 [[Abstract/Free Full Text](#)].
31. DeZazzo JD, Kilpatrick JE, Imperiale MJ. Involvement of long terminal repeat U3 sequences overlapping the transcription control region in human immunodeficiency virus type 1 mRNA 3' end formation. *Mol Cell Biol.* 1991;11:1624-1630[[Medline](#)] [[Order article via Infotrieve](#)].
32. Valsamakis A, Zeichner S, Carswell S, Alwine JC. The human immunodeficiency virus type 1 polyadenylation signal: a 3' long terminal repeat element upstream of the AAUAAA necessary for efficient polyadenylation. *Proc Natl Acad Sci U S A.* 1991;88:2108-2112 [[Abstract](#)].
33. Brown PH, Tilely LS, Cullen BR. Efficient polyadenylation within the human immunodeficiency virus type 1 long terminal repeat requires flanking U3-specific sequences. *J Virol.* 1991;65:3340-3343[[Medline](#)] [[Order article via Infotrieve](#)].
34. Cherrington J, Ganem D. Regulation of polyadenylation in human immunodeficiency virus (HIV): contributions of promoter proximity and upstream sequences. *EMBO J.* 1992;11:1513-1524[[Abstract](#)].



35. Valsamakis A, Schek N, Alwine JC. Elements upstream of the AAUAAA within the human immunodeficiency virus polyadenylation signal are required for efficient polyadenylation in vitro. *Mol Cell Biol.* 1992;12:3699-3705[[Abstract](#)].
36. Gilmartin GM, Fleming ES, Oetjen J. Activation of HIV-1 pre-mRNA 3' processing in vitro requires both an upstream element and TAR. *EMBO J.* 1992;11:4419-4428[[Abstract](#)].

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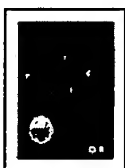
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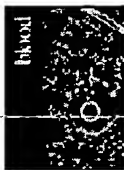
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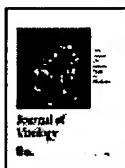
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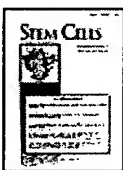
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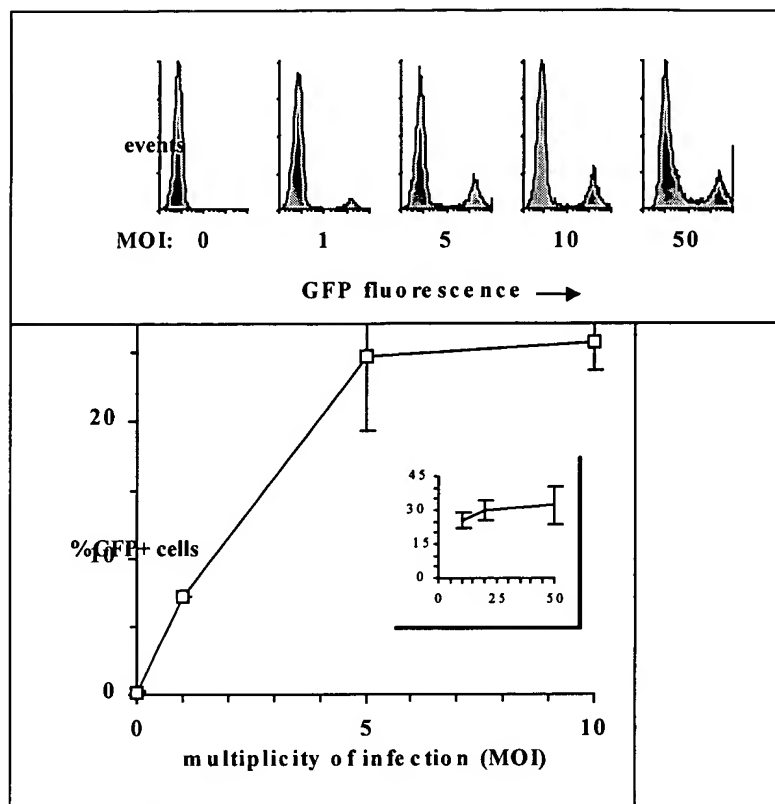
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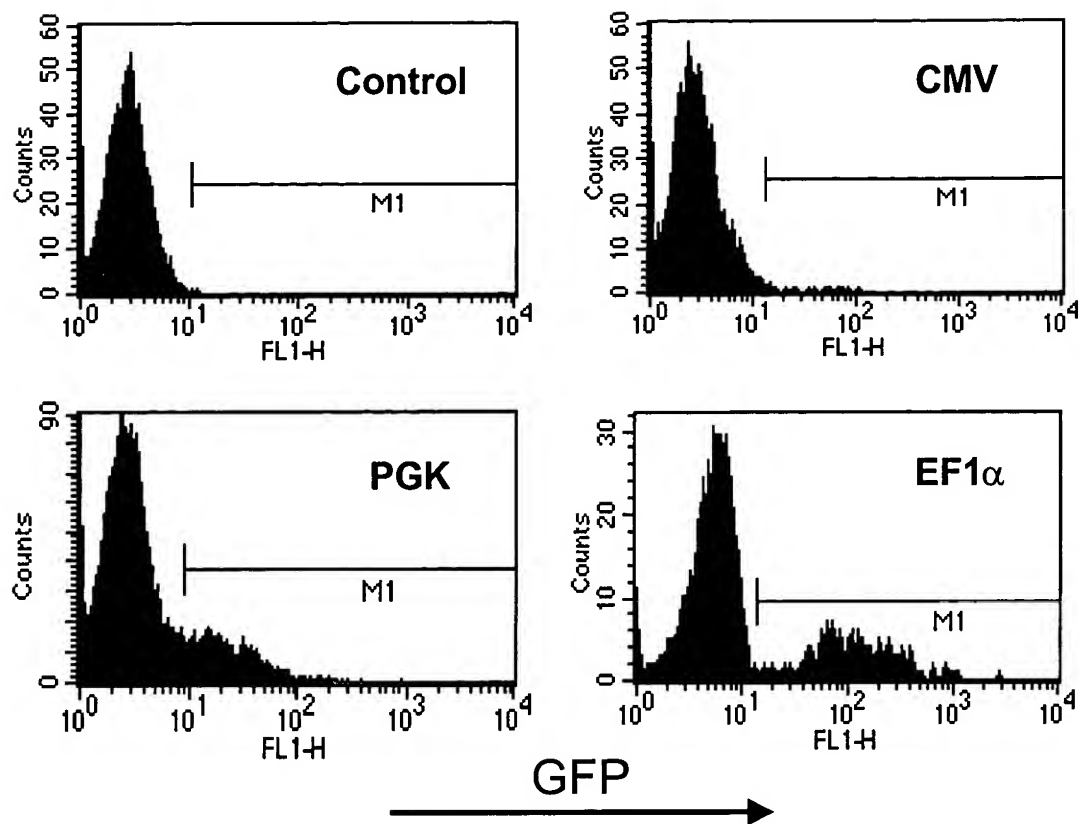
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P. Salmon et al., Figure 2





P. SALMON et al., fig4



# **High-level transgene expression in human hematopoietic progenitors and differentiated blood lineages after transduction with improved lentiviral vectors**

Running head: High-efficiency lentivectors for hematopoietic cells

(Abstract word count: 188)

(Total text word count: 4809)

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Recent experiments point to the great value of lentiviral vectors for the transduction of human hematopoietic stem cells (hHSC). Vectors used so far, however, have been poorly satisfying in terms of either biosafety or efficiency of transgene expression. Here, we describe results obtained with HIV-based vectors optimized in both of these aspects. We thus show that vectors containing the EF1 $\alpha$  and to a lesser extent the PGK promoter govern high level gene expression in human hematopoietic progenitors as well as derived hematopoietic lineages of therapeutic relevance such as erythrocytes, granulocytes, monocytes, dendritic cells and megakaryocytes. EF1 $\alpha$  promoter-containing lentiviral vectors can also induce strong transgene expression in primary T-lymphocytes isolated from peripheral blood. A self-inactivating (SIN) design did not affect the performance of EF1 $\alpha$  promoter-based vectors, but significantly reduced expression from the PGK promoter. This negative effect could nevertheless be largely rescued by inserting the posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE) upstream of the vector 3' long terminal repeat (LTR). These results have important practical implications for the genetic treatment of lympho-hematological disorders as well as for the study of hematopoiesis via the lentivector-mediated modification of hHSC.



## INTRODUCTION

Gene therapy via the transduction of human hematopoietic stem cells (hHSC) represents a very promising approach for the treatment of a number of inherited and acquired lympho-hematological disorders. The stable genetic manipulation of long term repopulating hHSC with existing gene delivery systems, however, was until recently impossible to achieve at an efficiency compatible with therapeutic realities. Oncoretroviral vectors derived from Moloney murine leukemia virus (MLV), for instance, although highly appealing since they integrate their cargo into the chromosomes of target cells, cannot transduce hHSC that have not been first treated with inducers of proliferation<sup>1,2</sup>. Indeed, the nuclear transport of the MLV preintegration complex requires the breakdown of the nuclear envelope that occurs at mitosis<sup>3,4</sup>. Unfortunately hHSCs, whether harvested from the bone marrow (BM), the umbilical cord (UC) or mobilized in the peripheral circulation, are mostly quiescent and lose their pluripotentiality after stimulation and proliferation<sup>5-7</sup>. Recent reports, however, have shown that a significant fraction of pluripotent cells as well as cells capable of long-term engraftment in non-obese diabetic/severe combined immunodeficient (NOD/SCID), also called SCID-repopulating cells (SRC), can be maintained, transduced and even expanded using specific stimulation conditions<sup>7-10</sup>.

Lentiviruses are a subgroup of retroviruses that can infect nondividing cells owing to the karyophilic properties of their preintegration complex, which allow for its active import through the nucleopore. Correspondingly, lentiviral vectors derived from human immunodeficiency virus type 1 (HIV-1) can mediate the efficient delivery, integration and long-term expression of transgenes into non-mitotic cells both in vitro and in vivo<sup>11-13</sup>. In particular, HIV-based vectors can efficiently transduce human CD34<sup>+</sup> hematopoietic cells in the absence of cytokine stimulation<sup>14-18</sup>, and these cells are capable of long-term engraftment in NOD/SCID mice<sup>17</sup>. Furthermore, bone marrow from these primary recipients can repopulate secondary mice with transduced cells, confirming the lentivector-mediated genetic modification of very primitive hematopoietic precursors, most probably bona fide stem cells (submitted). Since none of the other currently available gene delivery systems has such an ability, lentiviral vectors provide a previously unexplored basis for the study of hematopoiesis



and for the gene therapy of inherited and acquired lympho-hematopoietic disorders via the genetic modification of HSCs.

The demonstration of this important point, however, was provided with an early generation of lentiviral vectors unsuitable for therapeutic applications, either because they failed to meet biosafety requirements <sup>14-16</sup> or because they induced levels of transgene expression that were dismissingly low <sup>17-19</sup>. Our recent efforts have resulted in the development of lentiviral vectors improved in both of these aspects. Accordingly, the present article describes gene transfer vehicles that appear particularly well suited for the transduction of human hematopoietic precursor cells (HPCs) and for the expression of transgenes in differentiated blood lineages. Our results should facilitate the further use of lentiviral vectors for the genetic manipulation of lympho-hematopoietic cells, be it for research or therapeutic purposes.



## RESULTS

### **Transduction of human hematopoietic progenitors with MLV vectors and HIV-based lentivectors: The EF1 $\alpha$ promoter induces high transgene expression in lentivector-transduced CD34<sup>+</sup> cells.**

Human cord blood CD34<sup>+</sup> cells were purified by a single round of positive selection with magnetic beads, resulting in preparations approximately 90% pure. Cells were maintained overnight in serum-free medium supplemented with TPO, and were exposed for 24 hrs to MLV- or HIV-based GFP-expressing vectors pseudotyped with the G protein of vesicular stomatitis virus (VSV G), prepared by transient transfection of 293T cells as previously described<sup>11,20</sup>. The lentiviral packaging system used in these experiments was of a so-called second generation<sup>20</sup>, comprising the HIV-1 *gag*, *pol*, *tat* and *rev* genes. The multiplicity of infection (MOI) was 10 HeLa-transducing unit (TU) per CD34<sup>+</sup> cell. After transduction, cells were incubated for another three days in medium containing FCS, FLT3-L, TPO and SCF, and analyzed by flow cytometry for GFP (Fig.1) and CD34 expression (not illustrated) and lyzed for PCR analysis. The relative potencies of three internal transcription units were compared within the context of lentivectors: the cytomegalovirus immediate early (CMV), the phosphoglycerate kinase (PGK) and the alpha chain of elongation factor 1 $\alpha$  (EF1 $\alpha$ ) promoters. As shown in Fig. 1A, a sharp subpopulation of GFP-positive hematopoietic progenitors could only be seen when cells were transduced with HIV vectors containing the PGK or the EF1 $\alpha$  promoters. Cells transduced with MLV vector or HIV vector containing the CMV promoter displayed only a small percentage of GFP-positive cells, together with a high heterogeneity in GFP expression. A side by side comparison of GFP expression after transduction of HeLa and CD34<sup>+</sup> cells revealed that the promoters examined behaved quite differently in these two cell types. The PGK promoter was weak in HeLa cells (overlap between GFP<sup>+</sup> and GFP<sup>-</sup> cells) and strong in HPCs. The EF1 $\alpha$  promoter was intermediate in HeLa cells and very potent in HPCs, with a mean value typically 100 times higher in transduced than in control HPCs. The CMV promoter, whether in an MLV or an HIV vector, was equally strong in HeLa cells, contrasting with its low activity in transduced HPCs. A PCR-based quantification



performed at the time of the flow cytometry analysis (Fig. 1B) indicated that the low expression from the MLV-CMV vector in HPCs was due at least in part to poor gene transfer in these cells, a consequence of the limited stimulation of CD34<sup>+</sup> cells during transduction. In contrast, the low expression in HPCs exposed to the HIV-CMV vector (Fig. 1A) could not be explained by a low frequency of transduction, since equivalent amounts of transgene DNA were present in cells transduced with all three HIV vectors (Fig. 1B). This indicates that the CMV promoter does not govern efficient expression in hematopoietic progenitors, a finding in accordance with previous reports <sup>17-19</sup>. We also ascertained that GFP expression originated from integrated proviruses. For this, we performed a PCR analysis following 3 weeks of expansion of the transduced CD34<sup>+</sup> cells. During this time period, the cell numbers increased by a factor of 900 to 1800, depending on conditions and experiments. In the particular experiment illustrated here (Fig. 1B), the percentage of cells expressing GFP from the PGK promoter decreased by a factor of approximately 2 after expansion and differentiation into erythroid or monocytoid cells, which paralleled a slight decrease in GFP DNA copy number. It confirms that the bulk of transgene DNA remains in the cells after massive expansion, excluding its presence as unintegrated forms. It also correlates the previous demonstration that long term transgene expression cannot be detected after transduction with integrase-deficient HIV-vectors <sup>12</sup>.

### **Transduction of human CD34<sup>+</sup> cells as a function of increasing vector concentration**

The high levels of GFP expression induced by the EF1 $\alpha$ -containing HIV- derived vector allowed for a reliable determination of gene transfer efficiency, because even low numbers of transduced cells were easily detected. This vector was thus used to evaluate the influence of the MOI on the transduction efficiency of CD34<sup>+</sup> cell (Fig. 2). Although the percentage of GFP<sup>+</sup> cells initially increased as a direct function of the MOI, the curve flattened starting at a MOI of 5, reaching a maximum of 25 % GFP<sup>+</sup> cells ( $\pm 5\%$  according to experiments) at MOIs 20 and above. In the absence of optimal cytokine-induced proliferation, only a fraction of human CD34<sup>+</sup> cells is thus permissive to lentivector-mediated transduction, as previously suggested <sup>21</sup>.



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The differentiated cells were then analyzed by flow cytometry to determine the percentage of lineage-specific marker<sup>+</sup> / GFP<sup>+</sup> cells, as well as the relative levels of GFP expression in the differentiated populations (Fig. 3). Transgene expression was high in all lineages examined after transduction of precursors with the EF1 $\alpha$ -containing lentiviral vector, with a signal-to-noise ratio (mean of fluorescence intensity of GFP<sup>+</sup> cells divided by mean of fluorescence intensity of GFP<sup>-</sup> cells) comprised between 150 and 200 depending on the cell type. The PGK promoter induced lower and more variable levels of GFP expression in differentiated cells. Expression of GFP under the control of the PGK promoter was highest in DCs where it was only 3.5 less potent than that driven from the EF1 $\alpha$  promoter, and worst in erythroid cells (glycophorin<sup>+</sup>), where it was 11 fold weaker than its EF1 $\alpha$  counterpart. However, even in this



case, the signal-to-noise ratio was still high enough to permit a clear discrimination between GFP<sup>+</sup> and GFP<sup>-</sup> cells, allowing for the reliable analysis of transgene expression.

Taken together, these data indicate that the transduction of human CD34<sup>+</sup> cells with HIV-based vectors containing internal promoters derived from the EF1 $\alpha$  and to a lesser extent the PGK gene results in high levels of transgene expression in several hematopoietic lineages.

### **EF1 $\alpha$ promoter-based lentiviral vectors induce high levels of transgene expression in primary T lymphocytes**

The system used in these experiments did not allow for the easy differentiation of CD34<sup>+</sup> precursors into cells of the lymphocytic lineage. Therefore, to compare the relative value of CMV-, PGK- and EF1 $\alpha$ -containing HIV-derived vectors in these cells, mature T-lymphocytes purified from the peripheral blood were directly used as transduction targets (Fig. 4). The results revealed the same hierarchy as observed in HPCs and in the various other blood cell lineages, with the EF1 $\alpha$  promoter inducing levels of GFP expression significantly higher than those yielded by the PGK promoter, whereas cells transduced with CMV-based vectors exhibited levels of GFP expression that were incompatible with proper detection by flow cytometry. Of note, preliminary experiments in human primary B cells show that the CMV promoter is very active in these cells (not illustrated). This indicates that the poor activity of the CMV promoter is not universal in human hematopoietic cells.

### **Influence of SIN design and WPRE on transgene expression**

The use of self-inactivating (SIN) 3'LTR U3-deleted HIV vectors increases the biosafety of this system and avoids a possible interference between the viral LTR and the vector's internal promoter <sup>22</sup>. EF1 $\alpha$ - and PGK-containing HIV-derived SIN vectors were therefore tested for their ability to induce high levels of transgene expression in hematopoietic precursors (Fig. 5). Most interestingly, the SIN design was accompanied by a dramatic decrease (6 fold) in GFP expression within the context of the PGK vector, whereas it instead had a slightly yet reproducibly positive effect when introduced in the EF1 $\alpha$  vector. Inserting the sequence for



the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE)<sup>23</sup> upstream of the 3' LTR has been shown to promote transgene expression from HIV-derived vectors in some targets<sup>24</sup>. It indeed stimulated GFP production from the PGK promoter by a factor of 2.15 in hematopoietic precursors. In contrast, it exerted a negative effect on expression from the highly active EF1 $\alpha$  promoter, with a decrease by a factor of 1.85. Taken together, these indicate that the strong EF1 $\alpha$  promoter is less sensitive than the PGK promoter to a self-inactivating configuration, making it a better candidate for gene expression in hematopoietic cells.

## DISCUSSION

This work describes the transduction of human CD34+ cells with improved HIV-derived vectors aimed at increasing gene expression as well as biosafety. Our results indicate that these vectors can efficiently transduce CD34+ cells using conditions under which MLV-based vectors are inefficient. Furthermore, our study demonstrates that an HIV-derived vector containing an internal EF1 $\alpha$  promoter induces high levels of transgene expression in both hematopoietic precursors and in vitro differentiated blood lineages, as well as in primary T cells. Using this vector, we also found that human CD34+ cells can be efficiently transduced at a relatively low MOI, but that the efficacy of gene transfer saturates at about 20 to 30% of transduced cells. Moreover, the HIV-derived vector containing an internal EF1 $\alpha$  promoter fully tolerates a self-inactivating design.

Lentiviral vectors represent a very attractive tool for gene therapy of hematological disorders because of their ability to transduce pluripotent human hematopoietic stem cells<sup>14-18</sup>. Two essential issues will need however to be addressed prior to envisaging the use of HIV-based vectors for therapeutic purposes. The first issue is the biosafety, to which the use of multiply attenuated packaging constructs, stable producer cell lines and self-inactivating vectors will significantly reduce the risk that replication competent recombinants (RCR) emerge from the vector-manufacturing system. The second issue is the efficiency issue, i.e. the levels of transgene expression in the therapeutically relevant mature blood cell. Indeed, for



instance with hematological disorders resulting from an enzymatic deficiency or with anemias secondary to defects in hemoglobin synthesis, function will not be restored without achieving significantly high levels of expression of the curative gene. In that respect, GFP represents a marker of choice to study promoter activity in target cells, for it permits a direct quantification of the percentage of transduced cells as well as of the level of transgene expression in each individual cell.

CMV promoter-containing HIV-derived vectors can induce high levels of transgene expression in the central nervous system <sup>11-13</sup>, and have allowed the initial demonstration that pluripotent hematopoietic precursors can be efficiently transduced by this gene delivery tool. CMV-based vectors, however, are largely useless for transferring therapeutic genes into most lympho-hematopoietic cells, because in these targets their transcriptional activity is prohibitively low <sup>17-19</sup>. We thus observed that CMV-driven GFP production was insufficient to determine reliably either the percentage of transduced cells or the level of transgene expression in both CD34<sup>+</sup> cells and several differentiated hematopoietic derivatives (Fig. 1 and 4, and data not shown). In sharp contrast, vectors in which GFP was transcribed from the EF1 $\alpha$  or, to a lesser extent, the PGK promoter were highly active in both categories of cells.

The EF1 $\alpha$ -GFP lentivector was used to assess the dose responsiveness of CD34<sup>+</sup> cell transduction, since GFP<sup>+</sup> cells could easily be discriminated from GFP<sup>-</sup> cells even when present in very low amounts. Surprisingly, under conditions of limited stimulation, we found that increasing the MOI over 10 did not increase significantly the fraction of transduced cells, which plateaued between 20 to 30% depending on the experiments. A similar saturation of the fraction of transducible CD34<sup>+</sup> cells was previously noted, and attributed to the fact that lentivectors can transfer genes into cells that are in the G<sub>1</sub> but not in the G<sub>0</sub> phase of the cycle <sup>21</sup>. Still, lentivector-transduced CD34<sup>+</sup> cells are pluripotent<sup>17</sup> (and manuscript submitted). It remains to determine whether the fraction of permissive hHSC can be augmented, without triggering cell division, by the use of specific cocktails of cytokines, as recently described for the transduction of resting human T cells with HIV vectors <sup>25</sup>. Alternatively, transduction of human CD34<sup>+</sup> cells could also be improved under conditions promoting cell division, as recently reported <sup>7,10</sup>.



The MOI of 10 used here to achieve optimal transduction is significantly lower than the ones described in previous studies, where it ranged between 60-300 and 1000-3000<sup>17,18</sup>. This difference may be partly artefactual, because we define the titer of HIV vector stocks in HeLa cells, which are approximately 4 times less sensitive to transduction than 293T cells, another commonly used target for lentivector titration (data not shown). Nevertheless, it is also worth emphasizing that we expose the CD34<sup>+</sup> cells to the vector particles in a small volume (10<sup>5</sup> cells in 200  $\mu$ l) and during 24 hrs, two factors that could enhance the vector-target meeting probability.

Both HIV-PGK and HIV-EF1 $\alpha$  vectors were analyzed for transgene expression in differentiated hematopoietic cells after transduction of CD34<sup>+</sup> cells. GFP could be readily detected in all lineages analyzed, after transduction with both types of vectors. However, transgene expression in lineage marker-positive cells was consistently higher with the EF1 $\alpha$  promoter than with its PGK counterpart. Also, there was a lower degree of inter-lineage variation with the EF1 $\alpha$  promoter. It is remarkable that, with both of these promoters, the percentage of GFP<sup>+</sup> cells remained fairly constant as the CD34<sup>+</sup> cells proliferated (global increase in cell number comprised between 100 and 1000, see Fig. 3) and differentiated into their various hematopoietic derivatives. This indicates that there was neither silencing of the promoters nor preferential loss of transduced cells during the expansion and maturation phases of the culture. Engraftment and repopulation assays in NOD/SCID will be performed with both HIV-PGK and HIV-EF1 $\alpha$  vectors, to confirm the stability of expression from these promoters *in vivo*.

Lastly, we studied the effect of two modifications in the HIV vector backbone designed to improve its utility as a therapeutic tool. One is the deletion of a major part of U3 in the 3'LTR of the vector plasmid, leading to a self-inactivating (SIN) configuration<sup>22</sup>. This deletion reduces the potential for generation of replication-competent viruses and prevents potential interference between LTR and internal promoter. In some circumstances, however, it can negatively affect transgene expression, apparently by decreasing the efficiency of polyadenylation<sup>26-31</sup>. We found that the SIN design exerted a dramatically negative impact on GFP expression in CD34<sup>+</sup> cells transduced with a PGK-based lentivector, while it did not



decrease transcription efficiency from the EF1 $\alpha$  promoter. It is possible that the SIN deletion may be deleterious only in the context of low levels of transcription.

The SIN-induced decrease in PGK-driven transgene expression could be partly rescued by inserting the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) in the vector, immediately upstream of the deleted 3' LTR. This confirms our recent demonstration that WPRE can stimulate gene expression by a factor of 3 to 6 in epithelial cells<sup>24</sup>. However, the addition of WPRE did not stimulate GFP expression in CD34+ cells transduced with an EF1 $\alpha$  promoter-based vector, having instead a slight yet reproducible negative effect. Although our lack of understanding of the mechanism of action of WPRE makes it difficult to explain this difference, it could be that the high amounts of transcripts generated by the EF1 $\alpha$  promoter clog the pathway targeted by this post-transcriptional regulatory element.

In conclusion, the present work indicates that EF1 $\alpha$  promoter-containing HIV-derived vectors are both highly efficient and very potent for expressing transgenes in human hematopoietic progenitor cells as well as in all of their blood cell derivatives, even in a self-inactivating configuration. These vectors thus represent attractive tools for the genetic treatment of lympho-hematological disorders and for the study of hematopoiesis via the lentivector-mediated modification of human HSCs.



## MATERIALS AND METHODS

**Vectors.** Production of MLV- and HIV-derived vectors pseudotyped with the VSV G envelope protein was achieved by transient co-transfection of three plasmids into 293T epithelial cell line as previously described <sup>11</sup>. MLV vector particles were produced using the CMV-GagPol plasmid as packaging construct <sup>32</sup>, and a vector derived from pSLX, which expressed GFP from the CMV promoter. The HIV-derived packaging construct used was pCMV R8.91, which encodes the HIV-1 Gag and Pol precursors, as well as the regulatory proteins Tat and Rev <sup>20</sup>. VSV G was expressed from pMD.G. The HIV vector plasmids were derivatives of the original pHR' backbone <sup>11</sup>, with the following modifications. Self-inactivating vectors were produced from the previously described SIN-18 vector, which contains a deletion in the U3 region of the 3'LTR from nt. -418 to nt. -18, removing all the transcriptionally active sequences <sup>22</sup>. Insertion of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) between the reporter gene and the 3' LTR was done as previously <sup>24</sup>. The phosphoglycerate kinase (PGK) promoter corresponded to nucleotides 424 to 930 of the murine PGK gene (Genbank accession number M18735). The EF1 $\alpha$  promoter was derived from the pEF-BOS plasmid <sup>33</sup>, and comprised nucleotides 373 to 1561 of the human elongation factor EF1 $\alpha$  gene (Genbank accession number J04617). After transient transfection of the three plasmids by calcium phosphate in 293T cells, the supernatant was harvested, concentrated by 2 rounds of ultracentrifugation, and resuspended in serum-free Cellgro® SCGM medium (BioWhittaker Europe, Verviers, Belgium). Viral stocks were stored at -70° C and titers determined by transduction and flow cytometry analysis of GFP expression in HeLa cells as previously described <sup>20</sup>. Titers were comprised between  $5 \times 10^7$  and  $10^8$  HeLa-transducing units (TU) per ml.

**Purification and transduction of CD34<sup>+</sup> cells.** Cord blood (CB) samples were obtained according to institutional guidelines and CD34<sup>+</sup> cells were purified as described <sup>34</sup>. In brief, CB mononuclear cells recovered after Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation were incubated on ice with anti-CD34 M450 Dynabeads as described by the manufacturer. After several washes to eliminate unbound cells, CD34<sup>+</sup> cells were recovered



from the beads by incubation for 15 minutes at 37°C with the "Detach-a-bead" included in the kit. Cells were immediately washed, and analyzed by flow cytometry. The percentage of purified CD34<sup>+</sup> cells was  $89 \pm 7.0$ . For transduction,  $10^5$  cells were seeded in 96-well plates in 100 µl of Cellgro® SCGM medium supplemented with antibiotics (Gibco BRL, Life Technologies LTD, Paisley, Scotland, U.K.), with  $10^{-4}$  M dithiothreitol (Fluka Biochemika, Buchs, Switzerland) and TPO. After overnight incubation,  $10^6$  (typically), or  $10^5$  to  $5 \times 10^6$  (for dose-response analysis) HeLa-transducing units (TU) of vector were added per well, and the volume was adjusted to 200 µl with Cellgro® SCGM medium containing TPO. After 24 hours, cells were washed, diluted to 400 µl in IMDM supplemented with 10% of FCS (both from Gibco BRL, Life Technologies LTD), antibiotics, and FLT3-L, TPO and SCF for 3 days. Cells were either directly analyzed for GFP and CD34 expression, or further cultured with the 3 growth factors. For experiments shown in Fig.1, concentrated viral stocks were treated with DNase I prior to incubation with cells in order to eliminate DNA contaminants that may interfere with further PCR analysis. Briefly,  $10^6$  TU of vector was incubated for 30' at 37°C in a final volume of 20 µl containing 20 ug/ml Dnase I (DNase I, Roche Diagnostics, Rotkreuz, Switzerland) and 10 mM MgCl<sub>2</sub>. Then, 80 µl of Cellgro was added and the resulting 100 µl were added to 100 µl of Cellgro containing  $10^5$  CD34<sup>+</sup> cells. Cells were then cultured as described above.

**PCR analysis after transduction of CD34<sup>+</sup> cells.** Cells ( $5 \times 10^5$ ) were lysed in 18 µl of 50 mM Tris.HCl, pH 8.0, 20 mM NaCl, 1 mM EDTA, 1% SDS. Proteinase K (2 µl at 10 mg/ml) was added and samples were incubated for 30' at 55°C. After digestion, 200 µl of water was added and samples were boiled for 10' to inactivate proteinase K. PCR amplification was performed using HotStarTaq (Qiagen, GmbH, Hilden, Germany) in a final volume of 50 µl and 2 µl or 0.7 µl of extract with the following cycles: 95°C 15'; 40 cycles 94°C 30", 60°C 1', 72°C 1', plus 10' extension phase at 72°C. Sequences of the PCR primers were: 5' hIL2 gcaactcctgtcttcattg; 3' hIL2 aatgtgagcatcctggtgag;; 5'GFP gtgagcaaggcgaggagc; 3'GFP cttgatgccgttcttctgctgt. Conditions were adjusted to observe decrease in final PCR product yield when diluting samples by a factor of 3 (see Fig. 1A)



**Purification and transduction of primary human T Cells.** Peripheral blood mononuclear cells were purified from buffy coats of healthy donors over a Ficoll-Hypaque gradient. Macrophages were removed by plastic adherence for 2 hrs at 37° C, and the remaining cells were incubated with a cocktail of monoclonal antibodies against HLA-DR, CD25, CD69, CD19, CD16, CD11b and CD14 (Pharmingen). After a 30-min incubation on ice, cells were washed twice and incubated with magnetic beads (Dynabeads; Dynal, Oslo, Norway) conjugated with goat anti-mouse IgG, at a 1:4 target/bead ratio. 30 min later, bead-bound cells were removed using a magnet. Remaining cells were further purified through two more rounds at an increased target/bead ratio (1:10). This purification protocol typically resulted in a 99.5% pure population of resting T cells, as determined by flow cytometry analysis with antibodies against the activation markers HLA-DR, CD25 and CD69. Cells maintained in RPMI 1640 supplemented with 10% FCS, 5 mM penicillin and streptomycin (GIBCO BRL) and 2 mM glutamine (GIBCO BRL) were activated with phytohemagglutinin (Sigma) at 3 µg/ml for 48 hrs and subsequently cultured in RPMI 1640 containing 10% fetal calf serum and recombinant interleukin-2 (Sigma) at 10 U/ml.  $5 \times 10^5$  cells were transduced with various lentiviral vectors at a MOI of 5 HeLa-transducing unit per cell in 24-well plates in the presence of PHA, in a final volume of 500 µl. Flow cytometry analysis of GFP expression was performed five days after transduction.

**Cytokines.** All cytokines were recombinant human material. GM-CSF (Leucomax) from Essex Chimie & Sandoz (Basel, Switzerland) was used at 20 ng/ml, G-CSF (Neupogen) from Roche (Basel, Switzerland) at 10 ng/ml, and erythropoietin (Eprex) from Cilag (Schaffhausen, Switzerland), at 2U/ml. Other cytokines were purchased from Peprotech EC (London, U.K.) and used at the following concentrations: FLT3-L 25 ng/ml, TPO 10 U/ml, SCF 20 ng/ml, interleukin-3 10 ng/ml, TNF 40 ng/ml and IL-4 20 ng/ml.

**Antibodies/immunoreactants.** Phycoerythrin-conjugated monoclonal antibodies (Mab) were the following: anti-CD34 (mIgG1 clone 8G12) from Becton-Dickinson (Mountain View, CA), anti-glycophorin-A (mIgG1, clone JC 159), anti-CD42b (mIgG2a, clone AN51) an isotypic controls from Dako A/S (Glostrup, Denmark). Biotin-labeled mAbs: Anti-CD14 (mIgG2a, clone UCHM1) from Ancell Corp (Bayport, MN), and anti-CD15 (mIgM cloneDU-



HL60-3) from Sigma (St Louis, MO), and isotypic controls from Ancell. Allophycocyanin (APC)-labeled streptavidin was from Pharmingen (San Diego, CA). Anti-CD34 mIgG coated M450 Dynabeads were from Dynal A/S (Oslo, Norway).

**In vitro differentiation.** Megakaryocytic differentiation was evaluated after 10 days of expansion culture with FLT3-L, TPO, and SCF. For the other lineages, cells were washed after 9-11 days of expansion culture with FLT3-L, TPO, and SCF, and incubated with EPO, IL-3 and SCF for erythroid differentiation, GM-CSF and SCF for monocytic differentiation, and G-CSF and SCF for granulocytic differentiation. Differentiation into dendritic cells was performed as previously described <sup>34</sup>. Briefly, transduced CD34+ cells were expanded for 14 to 28 days with FLT3-L, TPO, and SCF. Cells were then induced into mature DC by exposure to GM-CSF and IL-4 for 3 days, followed by GM-CSF, IL-4 and TNF for 3 more days. The amplification ratio was comprised between 100 and 1000 depending on the experiment and the cell lineage.

**Flow cytometry analysis.** Cells were analyzed as described <sup>34</sup> on a FACScalibur (Becton-Dickinson) with slight modifications. FL-1 was used for GFP, FL-2 for PE-labeled mAbs, FL-3 for identification of living cells with the non-permeant DNA dye 7-amino-actinomycin D (Sigma) <sup>35</sup>, and FL-4 for biotinylated mAbs indirectly labeled with streptavidin-APC. Cell suspensions were adjusted to 0.5% paraformaldehyde prior to analysis. Data were analyzed using WINMDI software written by J. Trotter at Scripps Institute (La Jolla, CA) and CellQuest software (Becton-Dickinson).

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## REFERENCES

1. Kohn DB, Nolta JA, Weinthal J, Bahner I, Yu XJ, Lilley J, Crooks GM. Toward gene therapy for Gaucher disease. *Hum Gene Ther.* 1991;2:101-105
2. Mazurier F, Moreau-Gaudry F, Maguer-Satta V, Salesse S, Pigeonnier-Lagarde V, Ged C, Belloc F, Lacombe F, Mahon FX, Reiffers J, de Verneuil H. Rapid analysis and efficient selection of human transduced primitive hematopoietic cells using the humanized S65T green fluorescent protein. *Gene Ther.* 1998;5:556-562
3. Roe T, Reynolds TC, Yu G, Brown PO. Integration of murine leukemia virus DNA depends on mitosis. *EMBO J.* 1993;12:2099-2108
4. Lewis PF, Emerman M. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J. Virol.* 1994;68:510-516
5. Bhatia M, Bonnet D, Kapp U, Wang JC, Murdoch B, Dick JE. Quantitative analysis reveals expansion of human hematopoietic repopulating cells after short-term ex vivo culture. *J Exp Med.* 1997;186:619-624
6. Dao MA, Hannum CH, Kohn DB, Nolta JA. FLT3 ligand preserves the ability of human CD34<sup>+</sup> progenitors to sustain long-term hematopoiesis in immune-deficient mice after ex vivo retroviral-mediated transduction. *Blood.* 1997;89:446-456
7. Dorrell C, Gan OI, Pereira DS, Hawley RG, Dick JE. Expansion of human cord blood CD34(+)CD38(-) cells in ex vivo culture during retroviral transduction without a corresponding increase in SCID repopulating cell (SRC) frequency: dissociation of SRC phenotype and function. *Blood.* 2000;95:102-110
8. Dao MA, Hashino K, Kato I, Nolta JA. Adhesion to fibronectin maintains regenerative capacity during ex vivo culture and transduction of human hematopoietic stem and progenitor cells. *Blood.* 1998;92:4612-4621
9. Piacibello W, Sanavio F, Severino A, Dane A, Gammaitoni L, Fagioli F, Perissinotto E, Cavalloni G, Kollet O, Lapidot T, Aglietta M. Engraftment in nonobese diabetic severe combined immunodeficient mice of human CD34(+) cord blood cells after ex vivo expansion: evidence for the amplification and self-renewal of repopulating stem cells. *Blood.* 1999;93:3736-3749



10. Ueda T, Tsuji K, Yoshino H, Ebihara Y, Yagasaki H, Hisakawa H, Mitsui T, Manabe A, Tanaka R, Kobayashi K, Ito M, Yasukawa K, Nakahata T. Expansion of human NOD/SCID-repopulating cells by stem cell factor, Flk2/Flt3 ligand, thrombopoietin, IL-6, and soluble IL-6 receptor. *J Clin Invest.* 2000;105:1013-1021
11. Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science.* 1996;272:263-267
12. Naldini L, Blomer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. USA.* 1996;93:11382-11388
13. Blomer U, Naldini L, Kafri T, Trono D, Verma IM, Gage FH. Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J. Virol.* 1997;71:6641-6649
14. Akkina RK, Walton RM, Chen ML, Li QX, Planelles V, Chen IS. High-efficiency gene transfer into CD34<sup>+</sup> cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G. *J Virol.* 1996;70:2581-2585
15. Sutton RE, Wu HT, Rigg R, Bohnlein E, Brown PO. Human immunodeficiency virus type 1 vectors efficiently transduce human hematopoietic stem cells. *J Virol.* 1998;72:5781-5788
16. Uchida N, Sutton RE, Frieri AM, He D, Reitsma MJ, Chang WC, Veres G, Scollay R, Weissman IL. HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. *Proc Natl Acad Sci U S A.* 1998;95:11939-11944
17. Miyoshi H, Smith KA, Mosier DE, Verma IM, Torbett BE. Transduction of human CD34<sup>+</sup> cells that mediate long-term engraftment of NOD/SCID mice by HIV vectors. *Science.* 1999;283:682-686
18. Case SS, Price MA, Jordan CT, Yu XJ, Wang L, Bauer G, Haas DL, Xu D, Stripecke R, Naldini L, Kohn DB, Crooks GM. Stable transduction of quiescent



CD34(+)CD38(-) human hematopoietic cells by HIV-1-based lentiviral vectors. *Proc Natl Acad Sci U S A*. 1999;96:2988-2993

19. An DS, Wersto RP, Agricola BA, Metzger ME, Lu S, Amado RG, Chen IS, Donahue RE. Marking and gene expression by a lentivirus vector in transplanted human and nonhuman primate CD34(+) cells. *J Virol*. 2000;74:1286-1295

20. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol*. 1997;15:871-875

21. Sutton RE, Reitsma MJ, Uchida N, Brown PO. Transduction of human progenitor hematopoietic stem cells by human immunodeficiency virus type 1-based vectors is cell cycle dependent. *J Virol*. 1999;73:3649-3660

22. Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, Trono D. Self-inactivating lentivirus vector for safe and efficient In vivo gene delivery. *J Virol*. 1998;72:9873-9880

23. Donello JE, Loeb JE, Hope TJ. Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element. *J. Virol*. 1998;72:5085-5092

24. Zufferey R, Donello JE, Trono D, Hope TJ. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol*. 1999;73:2886-2892

25. Unutmaz D, KewalRamani VN, Marmon S, Littman DR. Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. *J Exp Med*. 1999;189:1735-1746

26. DeZazzo JD, Kilpatrick JE, Imperiale MJ. Involvement of long terminal repeat U3 sequences overlapping the transcription control region in human immunodeficiency virus type 1 mRNA 3' end formation. *Mol Cell Biol*. 1991;11:1624-1630

27. Valsamakis A, Zeichner S, Carswell S, Alwine JC. The human immunodeficiency virus type 1 polyadenylation signal: a 3' long terminal repeat element upstream of the AAUAAA necessary for efficient polyadenylation. *Proc Natl Acad Sci U S A*. 1991;88:2108-2112



28. Brown PH, Tilely LS, Cullen BR. Efficient polyadenylation within the human immunodeficiency virus type 1 long terminal repeat requires flanking U3-specific sequences. *J Virol.* 1991;65:3340-3343
29. Cherrington J, Ganem D. Regulation of polyadenylation in human immunodeficiency virus (HIV): contributions of promoter proximity and upstream sequences. *Embo J.* 1992;11:1513-1524
30. Valsamakis A, Schek N, Alwine JC. Elements upstream of the AAUAAA within the human immunodeficiency virus polyadenylation signal are required for efficient polyadenylation in vitro. *Mol Cell Biol.* 1992;12:3699-3705
31. Gilmartin GM, Fleming ES, Oetjen J. Activation of HIV-1 pre-mRNA 3' processing in vitro requires both an upstream element and TAR. *Embo J.* 1992;11:4419-4428
32. Scharfmann R, Axelrod JH, Verma IM. Long-term in vivo expression of retrovirus-mediated gene transfer in mouse fibroblast implants. *Proc Natl Acad Sci U S A.* 1991;88:4626-4630
33. Mizushima S, Nagata S. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* 1990;18:5322
34. Arrighi JF, Hauser C, Chapuis B, Zubler RH, Kindler V. Long-term culture of human CD34(+) progenitors with FLT3-ligand, thrombopoietin, and stem cell factor induces extensive amplification of a CD34(-)CD14(-) and a CD34(-)CD14(+) dendritic cell precursor. *Blood.* 1999;93:2244-2252
35. Schmid I, Uittenbogaart CH, Keld B, Giorgi JV. A rapid method for measuring apoptosis and dual-color immunofluorescence by single laser flow cytometry. *J Immunol Methods.* 1994;170:145-157



## FIGURE LEGENDS

### **Fig. 1. Transgene transfer and expression after transduction of human CD34<sup>+</sup> cells with MLV and HIV vectors.**

Human CD34<sup>+</sup> cells from umbilical cord blood ( $10^5$  cells) were transduced with  $10^6$  HeLa-transducing units (TU) of MLV- or the indicated HIV-derived GFP encoding vectors, previously treated with DNase I, as described in materials and methods. After 4 days, cells were analyzed by flow cytometry for GFP expression (**A**) and lysed for PCR analysis of the presence of GFP transgene (**B**). Results from one experiment representative of two independent evaluations are shown.

**A.** Expression of the GFP transgene after transduction of human CD34<sup>+</sup> cells or HeLa cells. Results are represented as histograms of GFP fluorescence intensity (x-axis, four-log scale) versus cell number (y-axis, linear). GFP<sup>+</sup> cells (within marker) were analyzed for percentage (lower number) and median of fluorescence intensity (upper number). Percentages were omitted in HeLa cells since the histograms were obtained from titration experiments of the corresponding vectors.

**B.** Presence of the GFP transgene after transduction and expansion of human CD34<sup>+</sup> cells. Cellular extracts equivalent to 5000 cells (upper panel) were amplified as described in materials and methods with GFP-specific primers, together with IL-2 specific primers as internal controls. Sizes of the corresponding PCR products are indicated. Lanes are: M, molecular weight marker; Hela (negative control for GFP, positive control for IL-2); 4.5, a clone of Hela containing one copy of HIV-CMV-GFP vector; 0, untransduced CD34<sup>+</sup> cells; MLV-CMV-PGK-EF1, CD34<sup>+</sup> cells transduced with the corresponding vectors (see A); CMV-EPO, CD34<sup>+</sup> cells transduced with HIV-CMV vector (lane CMV) and analyzed after expansion and differentiation into erythroid cells (see text); CMV-GM, same as CMV-EPO but analyzed after expansion and differentiation into monocytic cells (see text); PGK-EPO and PGK-GM, same as CMV-EPO and CMV-GM but from CD34<sup>+</sup> cells transduced with HIV-CMV vector (lane PGK). To ensure for proportionality, cellular extracts equivalent to 1700 cells (lower panel) were amplified separately.



**Fig. 2. Effect of MOI on transduction efficiency of lentivectors in human CD34<sup>+</sup> cells.**

10<sup>5</sup> CD34<sup>+</sup> cells were transduced with various doses (1, 2, 5, 10, 20 and 50x10<sup>5</sup> TU) of EF1 $\alpha$ -GFP lentiviral vector allowing multiplicities of infection (MOI) ranging from 1 to 50. GFP expression was analyzed by flow cytometry after 4 days. Upper panels: Data from one representative experiment are shown as frequency histograms of GFP fluorescence intensity versus cell number (events). Gates for GFP<sup>+</sup> cells were setup according to untransfected cells (MOI 0). Lower panel: Mean percentages of GFP<sup>+</sup> cells obtained with MOIs of 0 to 10 and 10 to 50 are represented in the main panel and in the insert respectively. These data are from 3 independent experiments for the 0-10 MOI range and from 2 for 10-50 MOI range. Error bars represent SD.



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